

The University of Chicago

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ATURES ON AVIAN MALARIAL
PARASITES

A DISSERTATION SUBMITTED TO THE FACULTY OF THE DIVISION OF THE BIOLOGICAL
SCIENCES IN CANDIDACY FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF BACTERIOLOGY AND PARASITOLOGY

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INTRODUCTION

Although much work has been done on the environmental modification of the periodicity of avian malaria by in vivo experiments, almost all of these attempts have involved the alteration of the environment or physiology of the avian host and have thus acted only indirectly on the malarial parasite. L. G. Taliaferro (1928), however, subjected strain 3H (the Hartman strain) of *Plasmodium cathemerium* to a temperature of 0.5 C in vitro. After inoculation into canaries the parasites were delayed in their asexual development in proportion to the time they had been subjected to the low temperature. The parasites shortened their reproductive cycle each day so that as the infections progressed they returned closer and closer to normal. In a short time the infections resulting from the cold treated parasites were indistinguishable from the control infections and did not deviate. With the exception of this report little work of this nature has been done on the parasite alone. Such an approach is of interest not only because of its bearing on the genetics of the malarial organisms but also because of the widespread interest in securing attenuated strains that would be feasible for use as a living vaccine. The present work was

done in an attempt to obtain a permanent modification of the characteristics of the parasites of strain 3H1-2 of *P. cathemerium* by subjecting the organisms to sublethal temperatures.

HISTORICAL REVIEW

The effect of environment on protozoa has been the subject of many investigations differing widely in their approach. In the free-living protozoa, prolonged heat over many generations, growth in different types of culture media, growth in the presence of weak solutions of toxic chemicals, and change in bacterial flora, have all resulted in physiological and morphological alterations in the organisms studied. In some instances the changes thus induced were permanent; in other cases, cultivation under the original cultural conditions caused a gradual reversion to the original characteristics. Jennings (1929, 1941) gives a detailed summary and analysis of the work on the free-living protozoa. The antigenic structure of the pathogenic trypanosomes can be altered by contact with antibody in a semiresistant host. Lysis of the majority of the trypanosomes present occurs. The remaining trypanosomes are found to be antigenically changed from their original composition and do not return to it as long as they remain in the particular host. A similar change of antigenicity as well as changes in morphology were noted in the production of drug "fastness." The drug "fastness" of trypanosomes has been found to continue for as long as 400 passages through the vertebrate host and in some cases

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at least several passages through the invertebrate host. W. H. Taliaferro (1926, 1929, 1941) and W. H. Taliaferro and Huff (1940) in a series of reviews give summaries of the work on the trypanosomes.

Since periodicity in avian malaria was first described by L. G. Taliaferro (1925), many papers have been written about the various factors influencing this phenomenon in both avian and monkey malaria. The Taliaferros (1934a) described a transient retardation of asexual development in *Plasmodium brasilianum* in monkeys after exposure of the parasites to refrigerator temperatures similar to that found by L. G. Taliaferro (1928) in infections with *P. cathemerium*. Lourie (1934) found that incubation of *P. cathemerium* with high concentrations of quinine in vitro had no effect on the subsequent infection.

All of the other papers concerning periodicity have dealt with the effect on the parasite while it was in the host. Boyd (1929a and b, and 1933), Gambrell (1937), and Stauber (1939) found that an alteration of the normal light-dark cycle of the host resulted in disturbance of the asexual cycle. The Taliaferros (1934b) by reversing the normal periods of dark and light were able to obtain a similar effect with *P. brasilianum*. Gambrell (1937) found that this extended as well to the periodicity of gametocyte development. Boyd (1933) considered disturbance of feeding habits as affecting periodicity, but Stauber (1939) did not find this to be a factor. The latter author found that exposure of the canary hosts to high temperatures at a time when the parasites were in the young ring state resulted in a disturbance of the synchronism of segmentation. Wolfson (1936) found that a loss of large amounts of blood apparently caused a disturbance of the regularity of schizogony. Lourie

(1934), Boyd and Allen (1934), and Boyd and Dunn (1939) found that the administration of quinine caused an almost complete loss of periodicity as well as an inhibition of schizogony. Those parasites that did reproduce had fewer merozoites per segmenter than normal. Hegner and West (1941a and b) inoculated 2 strains of *P. cathemerium* into ducks and fowls and found that in the duck the synchronism of infection progressively declined and that segmentation occurred later than it did in the control canaries. In the fowl the asexual cycle was lengthened from 24 to 48 hours with segmentation occurring much later in the evening than it did in the canaries. The number of merozoites per segmenter were fewer in the duck than in the canary. In both the abnormal hosts some of the gametocytes tended to become elongate and to encircle the red cell nucleus.

Immune phenomena also seem to be important in affecting the characteristics of malarial parasites. The Taliaferros (1939a) found that segmentation of *P. brasilianum* might be delayed and the synchronism disturbed at the crisis. These authors also found that in the marmoset the asexual cycle started regularly but soon became so irregular that segmenting forms were constantly present.

Huff and Gambrell (1934) noted that as a result of the rapid passage of strain "D" of *P. cathemerium* (strain 3D) over long periods of time, the gametocytes tended to diminish in number and finally were lost. By subinoculation from birds with latent infections which had had abundant gametocytes prior to the alteration in gametocyte production these authors were able to recover strains with the full complement of sexual forms. Two substrains completely lacking in gametocytes and one in which the gametocytes were scarce

arose during the rapid passage of the parent strains. Concomitant with the loss of gametocyte production, the parasites of these atypical strains exhibited a loss of synchronism in the asexual cycle. In a further study of these strains, Gambrell (1937) found that they had retained their lack of gametocytes and lack of synchronism for a period of 3 years. At the latter time Gambrell stated the strain "appears to be approaching a quotidian periodicity although the highest percentage of segmenting forms found was 18. These forms are more abundant at 24 hour intervals but segmenting forms are still present at all times." Another gametocyteless strain had been isolated in the meantime from strain "N" or Stauber's (1939) clone. This strain likewise exhibited a loss of periodicity in asexual development. For a discussion of the appearance of gametocyteless strains in human malaria, Gambrell's (1937) review of the literature should be consulted.

With the exception of the gametocyteless strain 3D1, and the Hartman-Hewitt's phenylhydrazine strain (3H3), none of the strains of avian malaria has undergone any permanent change as a result of experimental manipulation. When the factor causing the disturbance was removed the parasites returned to their normal character. Furthermore, with one of two exceptions, all of the attempts to modify the parasites or to delimit their characteristics have been indirect since procedures used affected only the avian host directly.

MATERIALS AND METHODS

All of the detailed studies in these experiments were made on strain "P" of *P. cathemerium*. This was Stauber's clone, which was transmitted by *Culex pipiens* after 30 blood passages by Huff (1939). The series of strain "N" infec-

tions, from which the strain "P" was thus derived, had almost lost the ability to produce gametocytes before the mosquito passage. Following the mosquito transmission the strain regained this ability. In accordance with the terminology of the Committee of Terminology of Strains of Avian Malaria (Huff, Boyd, Manwell, 1942), this strain will hereafter be designated as strain 3H1-2

The vertebrate hosts used throughout these studies were female canaries. In order to keep environmental conditions as uniform as possible, all infected birds were kept in the light-dark cabinet designed by Stauber (1939). The birds were kept in the light from 6 A.M. to 6 P.M., in the dark from 6 P.M. to 6 A.M. Blood smears were made in the usual manner by pricking a leg vein. When blood was needed for subinoculation, the desired amount was withdrawn directly from the jugular vein into a sterile 1 cc tuberculin syringe fitted with a 27-gauge needle. The feathers were plucked from around the neck of the bird and the site of the puncture sterilized with 70% alcohol. The needle was then slipped beneath the skin into the vein. In this manner it was possible to make an aseptic withdrawal of blood without the necessity of killing the bird. Heavily infected birds were used as donors whenever possible. However, in order to standardize the process, infected blood was mixed with heparinized physiological saline solution in such proportions that approximately 10 million parasites were found in 0.075 cc of the mixture. This number of parasites was used throughout the thermal death point experiments and, whenever a donor with a patent infection was available, in the subsequent experiments. If a donor bird had a latent infection, 0.25 cc of its whole blood was used for an inoculum. In determining the thermal

death points each bird was followed at intervals of not more than 2 days for 20 days. If no parasites were seen by the 12th day, 0.25 cc of whole blood was withdrawn and subinoculated into a clean bird. If no patent infection developed in either bird, the donor was adjudged uninfected. During the periodicity studies the birds were bled at 2-hour intervals from 8 A.M. until midnight. They were also bled at 4 A.M.

The first supplementary studies of the strains isolated in the thermal death point experiments were done 6½ months after the completion of the periodicity studies. Two birds were infected with parasites from the parent strain, and 2 from each of the strains isolated in the last sublethal temperature experiment of August 25, 1941. Four birds were inoculated, 2 from each donor, in the first supplementary study of the first strain isolated on May 1, 1941. A daily smear was made from each bird used in the first of these studies, throughout the course of the infections. The majority of these smears were made before segmentation had occurred, but a few were made after the peak of segmentation in order to determine the synchronism of the process.

A more intensive supplementary study was begun on April 15, 1942, seven months after the completion of the periodicity study. Since the characteristics of one of the heat treated strains, 3H1-5, had not shown any alterations as a result of the treatment, the 2 birds infected with this strain were used as controls. Two birds were inoculated with the parasites of heated strain 3H1-4, while 4 more birds were infected with parasites of heated strain 3H1-3. During this study the method of sampling was different. It was desirable to avoid causing an irregularity of the parasitic cycle by too much handling and loss of blood; yet it was equally

important to determine any changes that might occur in the synchronism of schizogony as the infections progressed. Observations were made at one or more of the following hours on each day of the infection: 12 P.M., 9-10 P.M., 12 M., 3 P.M., 6 P.M., and 9-10 P.M. A smear might be made from each bird at midnight, 9 A.M. and 12 M. on one day, and on the following day, at 6 P.M. and 9 A.M. However, a smear was made from each bird at each of the above times on at least 4 different days. Careful and complete studies from smears made at almost all of the above times, were made once during the early part of the infections, and once during the later part. By this method it was possible to determine whether the asexual cycle was at the expected stages of development at various hours during the day without the disorienting effect than an intensive periodicity study of the same length would have had on the parasites and their hosts. Variations noted in an individual bird could be compared with the data taken simultaneously from the other birds. They could also be checked with the data from the 2 more intensive studies made on the same bird. The progress of the various infections could thus be followed fairly well.

Culex pipiens, bred in the laboratory, was used in the mosquito infectivity experiments. Uninfected mosquitoes were allowed to feed on infected canaries following the method used by Huff (1927). Six days later the stomachs of the mosquitoes were dissected out and the numbers of oöcysts determined. No attempts were made to transmit the infection by mosquito bites or by sporozoite inoculation.

The pipettes, in which the parasites were exposed to heat in the thermal death point experiments, were drawn out in capillary form from clean glass

tubing. They were calibrated by weighing 0.075 gm of distilled water in them. The length of capillary tubing required to hold this volume was marked with a glass marking pencil. The tubes were then broken off at a distance of $1\frac{1}{2}$ inches or more from the markings and dried in a hot air oven. When used in the thermal death point determinations, a mixture of blood and heparinized physiological saline solution, containing approximately 10 million parasites in 0.075 cc was drawn up in each tube until the interval between the calibration marks was filled. The ends of the tube were sealed off in a flame and the preparation placed in a constant temperature water bath. The tube containing the blood mixture was withdrawn after the required interval and the outside sterilized with 70% alcohol. The ends were then broken off with forceps and the contents of the tube blown out into a sterile watch glass. In order to prevent any possible loss of viable parasites, heparinized saline was repeatedly drawn up into the tube and then expelled into the watch glass. The contents of the watch glass were further diluted by heparinized physiological saline solution and the final volume of approximately 0.4 cc mixed thoroughly before being drawn into the syringe. After the foot of the canary had been sterilized with 70% alcohol, the contents of the syringe were inoculated into the toe vein.

Gingrich's (1932) modification of the McNeal tetrachrome stain was used in staining the blood smears. His method of determining the probable error in the size of sample was also followed. However, instead of using the numbers of parasites per 10,000 red cells, the parasites are denoted by percentages of infected cells. Where the incidence of infected red cells was 1% or more, the probable error of the sample was 10%

or less; where the percentage of infected red cells was .5%, the probable error was 15% or less. Below an incidence of .5%, the probable error of the sample was 20% or less.

For clarity of future discussion and for interpretation of the tables, a series of definitions of terms used must be made. A young trophozoite was any small mononuclear parasite from a merozoite to a parasite whose cytoplasm was $\frac{1}{2}$ the size of a mature red cell nucleus. An old trophozoite was any parasite larger than the young trophozoite whose nucleus had not started to divide, or which was not obviously a pregametocyte. There were a few instances where a parasite was much larger than it ordinarily would be before the nucleus divided, and which also showed no evidences of sexual differentiation. Such parasites were classed with the pregametocytes. Young schizonts were parasites with 2 to 4 nuclei. Old schizonts were parasites with 5 or more nuclei, but less than 9. Segmenters were forms with 9 or more nuclei, or were actually in the process of cytoplasmic division. Gambrell's (1937) classification of sexual forms was followed. A total of at least 50 asexual parasites were thus differentiated in each smear made during the study. However, the percentage of segmenters was calculated from a total of at least 200 asexual parasites in order to obtain a count that would be more significant statistically.

THERMAL DEATH POINT EXPERIMENTS

Very little work has been done on determination of the thermal death points of species of *Plasmodium* in vitro. Coulston (1942) exposed strains 6A and 6E of *Plasmodium circumflexum* to temperatures of 40 to 55 C for periods of 5 to 30 minutes. A half-hour exposure to 40 C did not seem to affect the parasites. More than 15 minutes exposure to

TABLE 1.—*Survival of Parasites from Strain 3H1-2 of P. cathemerium After Exposure to Temperatures of 46–50 C for Varying Intervals of Time**

Length of Exposure in Minutes	Temperature				
	50 C	49 C	48 C	47 C	46 C
5	+(2) ^d – (1)	+(1) ^d	+(1) ^d	+(1)	+(1)
10	+(1) ^d – (1)	+(1) ^d	+(1) ^d	+(1)	+(1)
15	– (1)	+(1) ^s	+(1)	+(1)	+(1)
20	– (1)	– (1)	– (1)	+(1)	+(1)
25	– (1)	– (1)	– (1)	+(1) ^d	+(1)
30	– (1)	– (1)	– (1)	+(1) ^d	+(1)

+ indicates infection produced, – indicates no infection produced in birds receiving heat treated parasites. Numbers in parentheses indicate numbers of birds. ^d indicates an increase of 3 days or more in the prepatent period; ^s indicates that infection was discovered by subinoculation.

* Performed between November 15 and 23, 1940.

45 C resulted in a prolongation of the prepatent period from 3 to 6 days. No parasites survived after exposure to 50 C for 15 minutes or more, and after 5 and 10 minutes at this temperature, the incubation period was lengthened. When subjected to 55 C for 5 minutes, none of the parasites survived. Gingrich (1941b) used heat-killed *P. cathemerium* as a vaccine for immunization of canaries to the organism. Ampoules, containing heavily parasitized red blood cells, were immersed in a 50 C water bath. After the temperature in a control ampoule reached 50 C, the blood was allowed to remain for a period of 10 minutes. Approximately 40 to 60% of the red cells used in the preparation of this vaccine were parasitized.

Because of the small sizes of the capillary pipettes, the numbers of parasites used in the thermal death point experiments, described below, were much lower than those used by Gingrich and probably lower than those used by Coulston. The preparations were placed in capillary tubes because the thin walls of the glass and the large surface areas would cause practically immediate exposure of all the parasites to the same temperature. This procedure obviated the necessity of allowing time for the contents of the tubes to come to the temperature of the water

bath, and thus more sharply delimited the period of temperatures to which the parasites were subjected.

The results shown in table 1 demonstrated that the parasites would be killed by long exposure to lower temperatures, or by short exposure to higher temperatures. One infection resulted from blood that had been exposed for 10 minutes to 50 C. No infections resulted from inoculations of parasites which had undergone an exposure of 15 minutes or longer. Exposure to the same temperature for 5 minutes resulted in a slight prolongation of the incubation period. Subinoculation from the recipient of the treated parasites was necessary to recover an infection produced from parasites exposed to 49 C for 15 minutes. The point at which exposure to heat became deleterious or lethal was apparently very critical. Lowering the temperature as little as 1 degree necessitated prolonging the period of exposure 5 minutes or more in order to obtain an effect on the parasites. At 46 and 47 C none of the blood samples was sterilized, although after an exposure for 25 and 30 minutes, the time required to produce an observable infection was increased.

Table 2 summarizes the results from an attempt to determine accurately the length of exposure that would kill most, but not all, of the parasites. No infection was found in any of the birds which were inoculated with blood that had been exposed to a temperature of 50 C

TABLE 2.—*Survival of P. cathemerium (Strain 3H1-2) after Exposure to a Temperature of 50 C*

Length of Exposure in Minutes	Results of Inoculation of Heated Parasites into Birds
7	+(5), – (18)
8	– (8)
9	– (8)
10	– (8)

+ indicates infection produced; – indicates no patent infection produced; numbers in parentheses indicate numbers of birds.

for 8 minutes or more. There were surviving parasites in 5 out of 23 samples exposed for 7 minutes. Four of the infections had delayed incubation periods, but the other was not discovered until the bird was bled and 0.25 cc of its blood inoculated into an uninfected bird. In these 5 instances the majority of the parasites had undoubtedly been killed. Three of these strains were chosen for further study of the effects of heat on the parasites.

CHARACTERISTICS OF THE PARENT STRAIN, 3H1-2

Gametocyte production.—The largest number of mature gametocytes of this strain was found in the late evening and early morning hours during the period when the proportion of gametocytes to the total parasite count was lowest. Their number tended to decrease to its lowest point around 6 P.M. when the total number of sexual forms and the proportions of sexual to asexual stages were highest (See table 4). The macrogametocytes outnumbered the microgametocytes most of the time during the infections. The proportion between the two was 2:1 in the total count, and the times when the males predominated over the females were few and irregular.

The peak number of sexually differentiated forms, mature gametocytes and pregametocytes, combined, usually was found between midnight and 4 A.M. Their lowest total number occurred around noon. From noon until late evening the pregametocytes predominated over the mature sexual forms. The actual numbers of gametocytes counted were insufficient to give more than an indication of the trends. However, these observations confirm those of Gambrell (1937). In the overall picture of the parent strain, 3H1-2, the sexual stages formed a sizable proportion of the total, ranging from 3% to 35% of the total

parasite count depending on the time of day an observation was made. The sexual stages composed 14.2% of the total of 28,513 parasites of this strain counted during the periodicity study.

Periodicity in schizogony.—The data in fig. 2 and table 3 indicate a high degree of synchronism in the development of the asexual parasites in the parent strain. The largest number of segmenters was found at 8 P.M. in birds 385 and 386, and at 10 P.M. in bird 387. The peak numbers of the various asexual stages were found to occur somewhat later in bird 387 than they were in the other 2 birds. However, the periodicity of the parasites was sharp in all 3 birds since the time which elapsed between successive peaks of a given asexual stage was always 24 hours.

Segmentation was in general regular during the first 2 days of the experiment. During the third day a few segmenters were constantly present in bird 385, and in bird 386 except at 8 A.M. This was reflected by the fact that the numbers of young trophozoites did not decline as markedly on the last day. As a corollary, the number peaks of the asexual stages became lower—especially in bird 385. Study of the parasite numbers in the 3 birds gave a possible explanation for the differences in synchronism of development that were noted. In birds 385 and 386 the numbers of parasites failed to rise after the completion of segmentation at midnight of the 17th, and phagocytosis was accelerated during the last day of the experiment. It was apparent that these infections were undergoing a crisis during a major portion of the periodicity study. The Taliaferros (1934a), working with simian malaria, had found that during the crisis the synchronism of *P. brasilianum* was disturbed. Wolfson (1936) had found that the loss of large amounts of blood would cause a dis-

TABLE 3.—Percentages of Segmenting Parasites Throughout a 3-day Period in the Parent Strain of *P. cathemerium* and 3 Strains Derived from Heated Parasites

Date	Time	Parent Strain 3H1-2			Strain 3H1-3 From Heated Parasites			Strain 3H1-4 From Heated Parasites		Strain 3H1-5 From Heated Parasites		
		Bird No.			Bird No.			Bird No.		Bird No.		
		385	386	387	376	377	378	379	380	382	383	384
9/15	12 P.M.		2	7	2	2	2	4	1	1	2	0
	4 A.M.	0	2	2	1	2	0	3	1	0	1	0
	8 A.M.	0	0	0	3	2	1	0	1	0	0	0
	10 A.M.	0	1	0	3	2	2	0	0	0	0	0
	12 M.	0	0	0	3	1	0	1	0	0	1	0
	2 P.M.	1	0	0	6	4	0	0	0	0	1	0
	4 P.M.	2	0	0	5	3	3	0	1	0	0	0
	6 P.M.	15	18	5	11	8	10	3	1	3	5	13
	8 P.M.	14	32	36	6	8	5	6	2	44	28	40
	10 P.M.	4	16	50	2	10	7	12	4	9	11	19
9/16	12 P.M.	2	4	9	0	3	1	6	3	1	2	1
	4 A.M.	0	1	1	1	3	1	3	2	0	0	1
	8 A.M.	0	0	1	4	0	2	1	2	0	0	0
	10 A.M.	1	1	0	3	1	0	1	1	0	0	0
	12 M.	3	1	0	14	4	4	0	2	0	0	0
	2 P.M.	1	1	1	10	4	8	1	1	1	1	0
	4 P.M.	2	1	0	9	4	14	1	1	0	0	1
	6 P.M.	9	10	2	9	9	9	2	2	13	5	5
	8 P.M.	26	19	38	8	3	9	4	1	44	23	33
	10 P.M.	17	13	50	5	0	8	12	2	2	26	7
9/17	12 P.M.	2	8	18	2	3	5	6	2	0	0	1
	4 A.M.	1	3	2	4	1	1		1	0	0	
	8 A.M.	1	0	0	0	2	4	0	1	0	1	0
	10 A.M.	1	1	1	6	3	1	0	0	0	1	0
	12 M.	0	2	0	7	6	5	1	2	0	0	0
	2 P.M.	1	2	1	6	4	4	0	0	0	0	0
	4 P.M.	4	2	1	4	4	7	0	2	0	0	0
	6 P.M.	9	9	2	6	7	4	0	1	21	1	11
	8 P.M.	17	27	9	9	7	8	2	2	41	21	42
	10 P.M.	9	8	21	8	4	8	3	6	2	12	2

Based on a count of 50 asexual parasites

TABLE 4.—Percentages of Gametocytes Throughout a 3-day Period in the Parent Strain of *P. cathemerium* and 3 Strains Derived from Heated Parasites

Date	Time	Parent Strain 3H1-2			Strain 3H1-3 From Heated Parasites			Strain 3H1-4 From Heated Parasites		Strain 3H1-5 From Heated Parasites		
		Bird No.			Bird No.			Bird No.		Bird No.		
		385	386	387	376	377	378	379	380	382	383	384
9/15	12 P.M.		9	3	11	13	12	1	0	15	20	8
	4 A.M.	13	4	3	12	12	9	1	0.5	15	10	6
	8 A.M.	5	6	4	12	14	10	0	0	10	11	7
	10 A.M.	9	10	7	14	14	13	0	0	16	15	13
	12 M.	10	16	8	11	14	15	0	0	23	19	19
	2 P.M.	16	15	18	18	18	15	0.5	0	33	30	30
	4 P.M.	21	25	9	17	26	17	2	1	33	40	44
	6 P.M.	23	29	26	10	20	25	3	1	46	50	49
	8 P.M.	22	31	25	14	21	20	4	0.5	51	60	43
	10 P.M.	13	18	23	13	21	26	2	0	25	18	31
9/16	12 P.M.	8	13	7	17	13	11	3	0.6	20	10	19
	4 A.M.	14	6	4	13	12	11	0.5	0	8	10	14
	8 A.M.	8	8	2	10	5	6	0	0.6	6	13	13
	10 A.M.	14	9	3	16	19	8	0	0	8	15	10
	12 M.	22	11	7	21	19	20	0	0.5	17	18	13
	2 P.M.	15	22	8	32	11	15	1	0	23	40	16
	4 P.M.	20	15	13	15	10	15	2	0.3	30	36	22
	6 P.M.	26	13	20	20	26	22	2	0.6	50	30	31
	8 P.M.	35	19	18	21	23	14	2	0.6	51	50	47
	10 P.M.	18	14	30	21	16	9	3	0.7	23	43	20
9/17	12 P.M.	18	14	21	14	11	14	0.5	1	9	15	10
	4 A.M.	11	7	8	15	15	9	2	1	8	9	
	8 A.M.	14	8	5	21	13	11	0	1	6	10	4
	10 A.M.	15	8	9	17	15	13	0.5	0.2	10	16	11
	12 M.	19	17	10	22	11	14	0	0	12	26	13
	2 P.M.	21	18	12	23	26	20	1	0	17	25	17
	4 P.M.	17	18	11	21	22	24	0.5	1	43	36	41
	6 P.M.	17	14	19	18	20	23	2	0.4	15	27	46
	8 P.M.	22	18	17	13	24	22	0.5	1	45	37	44
	10 P.M.	17	15	9	12	19	26	0.5	0.5	13	20	12

turbance in the synchronism of malaria. Crisis and blood loss, coupled with the repeated disturbances through the night, probably were sufficient to account for the tendency of these infections to become asynchronous. In spite of this tendency, however, these infections were still highly synchronous during the last day of observation.

The infection in bird 387 rose continuously during the experiment, and there was no indication in the last smear made that the infection would not continue to increase. As a matter of fact, this bird died of the infection at 10 P.M. on the following day with a parasitemia of 55% at 8 P.M. The infections in the other 2 birds were less than 1% at this time.

In this study as well as from observations made 6½ months later (birds 408 and 409) it was found that the parent strain, 3H1-3, had a high degree of synchronism in asexual reproduction and had the ability to produce large numbers of gametocytes.

CHARACTERISTICS OF HEAT TREATED STRAIN, 3H1-3

Gametocyte production.—As shown in fig. 1 this strain resulted from the parasites recovered in one of the 2 birds which became infected of the 8 inoculated with heated parasites on May 1, 1941. In intensive studies made on this strain September 15 to 17, 1941, it was found that the percentage of gametocytes in the total parasite count was about the same as was found in the parent strain since the sexual forms composed 16% of the total of 25,000 parasites counted. However, there was no periodicity in the production of the sexual forms comparable to that seen in the parent strain (See table 4). Interestingly enough these infections were the only ones in which the males predominated over the females. Pregame-

toytes predominated over the mature gametocytes through most of the experiment.

An additional study was made of this strain 6½ months later (birds 410–413). The data, which are difficult to show graphically, are recorded elsewhere (Caldwell, 1943). In general they show

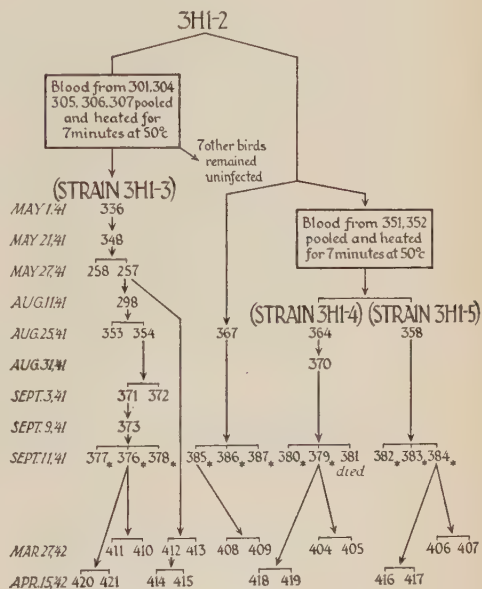


Fig. 1.—Genealogy of strains. Asterisks indicate the birds used in the periodicity studies shown in fig. 2.

that the synchronism of gametocyte production characteristic of the parent strain had returned at this later date.

Periodicity in schizogony.—Two studies were made of the periodicity of this strain, one of them 76 days and another approximately 9 months after isolation. The data from the first series are summarized in table 3. They reveal that a profound change had occurred in the periodicity of this strain as compared with that of the parent strain.

Throughout the experiment all stages of asexual development were observed. However, each asexual stage was slightly more numerous during a certain

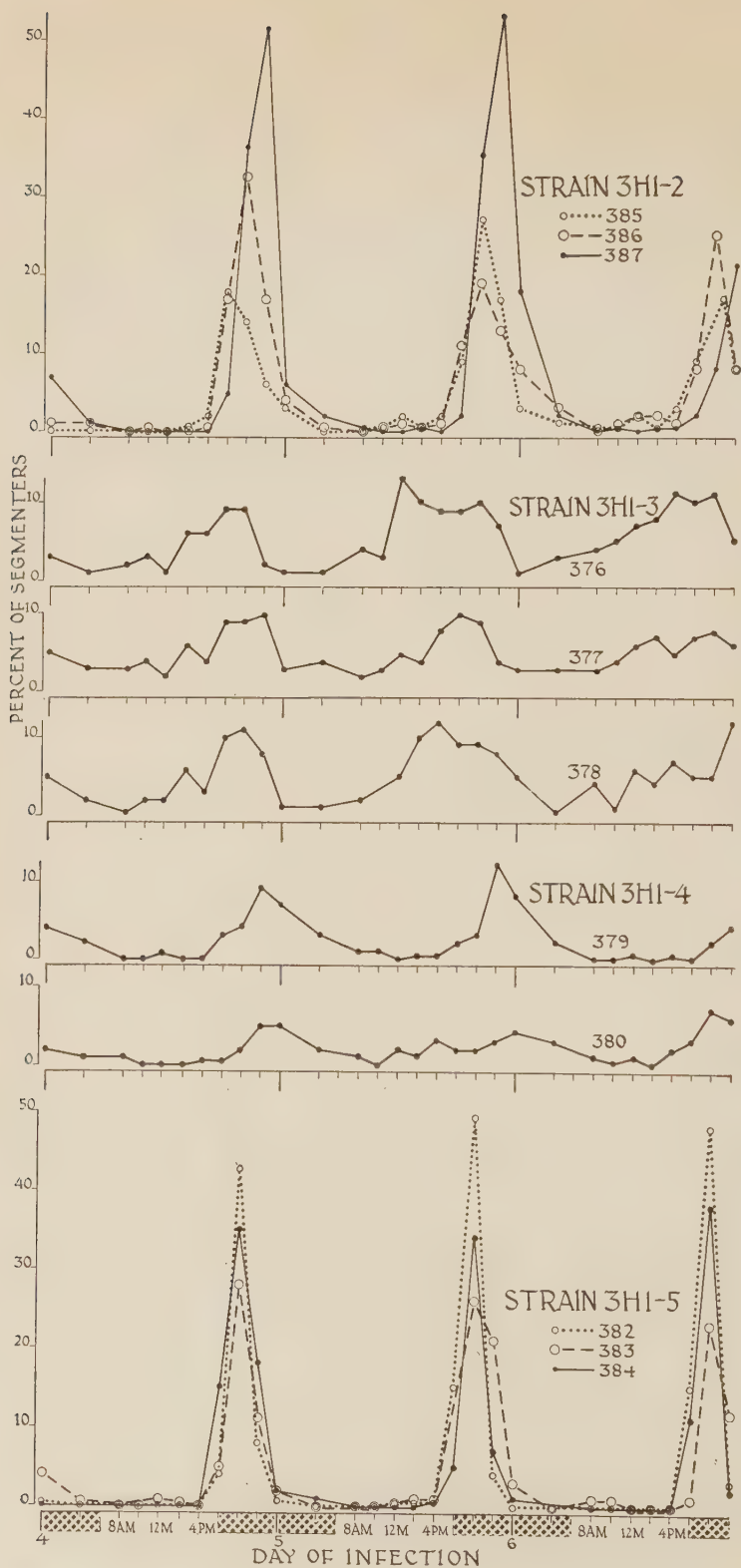


Fig. 2.—Graphs of percentages of segmenters in parent strain and 3 strains derived therefrom, drawn from data obtained from samples of 200 parasites from each blood smear.

period of each day. The peak numbers of several stages were frequently concurrent, and there was no sequential development in which the maximum number of one stage was followed by the maximum number of the next stage. Large numbers of asexual parasites in other phases of development were present even during the period when one form was at its maximum. Consequently the number peaks were low.

The effect of the lack of synchronism on the periodicity of segmentation is brought out in fig. 2. Segmenting parasites were more numerous from noon until midnight and formed a long low peak of 13% or less. Nevertheless, they composed 2 to 5% of the total asexual parasites during the hours when they were at their minimum. Because of this lack of periodicity in segmentation the occurrence of the maximum total numbers of parasites was also irregular. It is possible that some of this change was brought about by the beginning of an immune response in birds 376 and 377. This cannot be said for the infection in bird 378. Furthermore, the amount of disturbance in these infections was much greater than that seen in other birds whose infections were probably undergoing a crisis during the periodicity experiment. It seemed safe to assume, therefore, that the differences displayed by the parasites of strain 3H1-3 were definitely due to the treatment by heat. However, it seemed desirable, in view of these findings, to supplement the periodicity studies with further intensive studies on the parasites of this strain.

The results of the supplementary studies which, as indicated above, were made about 9 months after isolation of the strain are given in detail elsewhere (Caldwell, 1943). They reveal that strain 3H1-3 had returned to normal in the $6\frac{1}{2}$ months interval between the

periodicity study and the beginning of the supplementary studies. The infections in all 8 birds were highly synchronous in development with regard to both sexual and asexual parasites. Segmentation occurred between 8 and 10 P.M. and was usually completed by midnight. There was little evidence of a disturbance of the synchronism of segmentation at any time in birds 410 to 413. However, development of the parasites was slightly delayed in bird 414 at the time of the crisis and was markedly retarded just before the deaths of birds 414, 415, and 420. Similarly a delay was noted in the developmental cycles of the infections in birds 420 and 421 during or just after the number crisis. Concomitant with this delay in development there was a marked disturbance in synchronism of segmentation of the parasites which affected the sexual as well as the asexual forms. Synchronism was restored and the parasites returned to their previous schedule of development after the crisis was terminated. In none of the infections was the synchronism of segmentation as disturbed as it had been during the periodicity study.

From the study of these infections it would seem that the heat treatment had caused marked disturbances in the synchronism of schizogony of the parasites, which were still in effect 3 months after the isolation of the strain. During a further interval of $6\frac{1}{2}$ months, the strain had returned to normal.

CHARACTERISTICS OF HEAT TREATED STRAIN, 3H1-4

Gametocyte production.—The derivation of this strain is shown in fig. 1. After its isolation it had been passed through one canary before being used to infect birds 379 and 380 for the first series of observations. As shown in table 4 there was a striking difference be-

tween this and the parent strain in gametocyte production. Sexual forms did not constitute more than 1% of the total parasite population in bird 380, nor more than 4% in bird 379 and were completely absent in many of the smears. Twenty-three of the 87 gametocytes were counted as pregametocytes merely because they were large uninuclear forms which should have shown divided nuclei by the time their cytoplasm had attained such a size. Except in size these questionable pregameto-

TABLE 5.—Ability of Strain 3H1-4, As Compared With Parent Strain 3H1-2, to Infect Mosquitoes

Lot	% of Infected Red Cells	% of Red Cells Infected by Gametocytes	Number of Mosquitoes Dissected	Number of Mosquitoes Positive
Strain 3H1-2				
1	5.5	.55	11	9
2	11.2	2.46	3	3
3	11.8	2.36	8	6
4	16.5	1.65	4	4
			26	22
Strain 3H1-4				
1	11	0	5	0
2	19.4	0	3	0
3	13	0	3	0
4	10.1	0	8	0
			19	0

cytes showed none of the characteristics of sexual differentiation. However, including these forms, the gametocytes composed 0.7% of the total of 15,611 parasites studied in the strain, as compared to 15% of the total parasite population in the parent strain.

Two supplementary series of observations made 6½ and 7 months later respectively revealed no mature gametocytes of any type and only 3 questionable pregametocytes out of 13,400 parasites counted.

Since a highly susceptible strain of mosquitoes offers a delicate means of testing for the presence of gametocytes not evidenced by blood examination, this method was used to determine whether or not there were gametocytes present in strain 3H1-4. Each of 4 lots

of laboratory bred *Culex pipiens* was separated into 2 equal groups. The groups, fed on birds infected with the parent strain, 3H1-2, were used for controls. The other groups were fed on birds infected with strain 3H1-4. The mosquitoes were dissected on the 6th day after feeding, and their stomachs examined for oöcysts by wet preparations. As may be seen by the results summarized in table 5, 84.6% of the mosquitoes fed on the parent strain were positive. None of the mosquitoes fed on the heat treated strain had oöcysts. The numbers of mosquitoes tested were small and many died before dissection. Nevertheless, since the lots had been mixed, the average susceptibility should have been the same. The results seem to indicate that functional gametocytes were absent from strain 3H1-4.

Periodicity in schizogony.—The asexual synchronism of the development of the parasites of strain 3H1-4 was markedly disturbed and was only roughly periodic in comparison with that of the parent strain (See table 4). The consistent presence of large numbers of uninuclear parasites was indicative of the amount of asynchronous segmentation. The numbers of parasites in a given stage of development were slightly more numerous during one long period of the day than at others, thus forming a number "plateau." The variations in numbers of parasites within these "plateaux" were insignificant. Because of this lack of synchronism the maximum percentages of a given stage of parasite were low. The periodicity of segmentation of these parasites in birds 379 and 380 is shown in fig. 2. Asynchronous segmentation was constantly in evidence in both birds, but was more marked in bird 380. The numbers of segmenters did not rise to more than 12% in bird 379, nor more than 5% in

bird 380—a fact which indicated the marked loss of synchronism in the parasites of this strain. There was little likelihood of this irregularity of the asexual cycle being due to immune phenomena because the infections in both birds were still increasing at the end of the periodicity experiment.

The data from the two supplementary studies made $6\frac{1}{2}$ and 7 months later respectively are not suitable for graphic portrayal and since they are given in detail elsewhere (Caldwell, 1943), they will not be included here. In brief, they indicated that the characteristics of the asexual development were essentially the same as they had been during the first series of observations, i.e., the synchronism of reproduction remained markedly disturbed.

In summary, strain 3H1-4 which resulted from heating parasites of strain 3H1-2, after only one passage through canaries, showed pronounced disturbance in periodicity of asexual parasites and marked reduction in ability to produce gametocytes. After $6\frac{1}{2}$ or 7 more months the marked disturbance in periodicity of schizogony persisted. Moreover, at this time the gametocytes had been reduced to a very small number of questionable forms, and attempts to infect *Culex pipiens* were unsuccessful.

CHARACTERISTICS OF HEAT TREATED STRAIN, 3H1-5

As shown in fig. 1, this strain was obtained from the same thermal death point experiment in which strain 3H1-4 was isolated. Neither in the periodicity studies carried out 3 weeks after the isolation of the strain nor in those done $6\frac{1}{2}$ months later was there any indication that in this strain there had been any change in the periodicity of segmentation nor in the ability to produce gametocytes as compared with the parent strain. The increase in percentage

of gametocytes from 14 for strain 3H1-2 to 27 for strain 3H1-5 is well within the normal variability of a strain in its ability to produce gametocytes. Data for this strain on periodicity of asexual reproduction and ability to produce gametocytes are shown in fig. 2 and tables 3 and 4. It is apparent from these observations that neither the high degree of synchronism of asexual development nor the gametocyte production had been altered as a result of the heat treatment.

DISCUSSION

Shah (1934) and Gambrell (1937) found that the production of gametocytes in *P. cathemerium* occurred periodically at, or around the time of segmentation; Gambrell found that the gametocytes did not mature until several hours later and evidently were the products of the previous segmentation. When the day and night schedule of the avian host was reversed, the periodicity of both gametocyte production and asexual reproduction was reversed. Prolonged exposure to light caused asynchronous development of both forms of the parasite. On the basis of these findings and in order to account for the periodicity of gametocyte production, Gambrell postulated that gametocytes are derived from undifferentiated merozoites which are capable of becoming schizonts or gametocytes. Her findings are corroborated by data in the present study in which all environmental factors that might affect the periodicity of the parasites were kept constant. When the development of the asexual parasites of strain 3H1-3 were almost completely asynchronous, there was an equal disturbance of the periodicity of the gametocytes. In the later studies, when strain 3H1-3 had regained its periodicity, both asexual and sexual forms showed synchronous develop-

ment. The Taliaferros (1934a) had found a temporary disturbance of the periodicity of *P. brasilianum* of the monkey during the crisis. During the crises of some of the infections studied in the course of the present work, several types of transient disturbance were noted. Sometimes there was a delay in the normal time of segmentation. At other times, there was a lack of synchronism in the process of schizogony. These might occur separately, or simultaneously. In either case there was a similar derangement of the gametocyte production.

An apparent association exists between the ability to produce gametocytes and the synchronism of asexual reproduction in *P. cathemerium*, as is evidenced by the results of Huff and Gambrell (1934), Gambrell (1937), and the present study. Unfortunately no data are available concerning the periodicity of the parasites of strain 3D1 at the time immediately preceding the loss of its powers of gametocyte production. Figures, regarding the synchronism of asexual reproduction in those birds inoculated with parasites belonging to the strain characterized as "gametocyte poor," are likewise not available. The infection of their bird 247 (See fig. 2 of Huff and Gambrell, 1934), which had no gametocytes and exhibited a marked asynchronism of asexual reproduction, served as the source of the "gametocyte poor" strain, as well as the agametocyte strain, 3D1 (See fig. 1, Gambrell, 1934). At least a few of the parasites derived from their bird 247 were able to produce moderate numbers of gametocytes. The relation of gametocyte production and synchronism of the strain might be more clearly elucidated if periodicity data were available for these birds.

Gambrell interpreted the greater regularity of the course of the infection in

her bird 129F (See fig. 5, Gambrell, 1934) in comparison with that of 34F, as evidence that the agametocyte strain was regaining a quotidian periodicity. It should be noted that in the periodicity study of strain 3H1-4 (See fig. 2) the graphs of these parasites were similar to those of Gambrell's birds noted above. However, the parasites of bird 379 were more synchronous than those of 380 and their periodicity was similar to that of Gambrell's bird 129F. The graph of the parasites of bird 380 of strain 3H1-4 was similar to that of Gambrell's 34F. In spite of the fact that all subsequent infections with strain 3H1-4 were derived from bird 379, the infections of birds 404 and 418 were more synchronous than those of 405 and 419. The apparently close relationship between the ability to produce gametocytes and the sharp synchronism of asexual reproduction in *P. cathemerium* does not extend to some of the other species of avian malaria. In *Plasmodium relictum*, for instance, there may be an abundant gametocyte production but a relatively asynchronous asexual reproduction.

The ability to produce gametocytes must be an inherent quality of the malarial parasite and this ability must reside in a single undifferentiated individual, since both asexual and sexual parasites were derived in a clone (Stauber, 1939). This makes an explanation of the origin of the agametocyte strains rather difficult unless, as Gambrell pointed out in regard to her strains, a simultaneous mutation had occurred in large numbers of the organisms. In the cases of the free-living protozoa (See reviews by Jennings, 1929, 1941) and of the trypanosomes (See reviews by W. H. Taliaferro, 1926, and W. H. Taliaferro and Huff, 1940) the subjection to severe environmental stress, which has a highly selective action, is

usually correlated with a hereditary change of an organism. The natural immunity of birds to infections with avian malaria might be such a selective factor under certain circumstances. A large number of the parasites fail to develop to maturity during the period of the acute rise of the infection (L. G. Taliaferro, 1925; Hartman, 1927; Boyd and Allen, 1934; Boyd, 1939). Cannon and W. H. Taliaferro (1932) were able to correlate the disappearance of the parasites during the acute rise of infection with the phagocytosis of parasitized erythrocytes. However, Gingrich (1941a) obtained evidence that the phagocytes ingested parasites which had probably been injured by the factors of natural immunity.

With a tremendous loss of parasites due to the mechanisms of natural immunity in each infection, selection undoubtedly comes into play. In the case of the origin of the agametocyte strain, 3D1, of Huff and Gambrell (1934), it seems that natural immunity might be playing a more heightened role than normally. The parasites were uninterruptedly exposed for long periods of time to the natural immunity of many birds during the rapid passages made by these authors. The organisms had no chance to develop normal infections (they were immediately patent) or to adapt themselves to one host, before being inoculated into another host. This possibly might be the unfavorable factor in the environment of the organisms which might lead to a mutation of the characteristics of strain 3D. In the present experiments heat was apparently selective in its action on the parasites of strain 3H1-2 in the capillary tubes. This action was evidenced by the low numbers of parasites and long incubation periods in those birds that were infected by the surviving heat-treated parasites. A glance at table 2 shows that

the majority of the parasites in these experiments did not survive at all. It is not clear what factor or factors might have contributed to the mutation (or alteration of inherited characteristics) of strain 3H1, that resulted in the atypical strain found by Gambrell (1937).

Whatever the actual cause might be of the origin of the agametocyte strains of Gambrell, it appears that the change in characteristics of strain 3H1-3 were of a nature similar to Jollos' "Dauermodifikationen" in the free-living protozoa (1921). The asynchronism of the reproductive cycle, both asexual and sexual, lasted for at least 120 asexual generations. This was much longer than any previously produced alterations, all of which disappeared when the causative stimulus was removed. Another type of long lasting but transient, change has been found by Thompson and Huff (1944). These authors found that *Plasmodium mexicanum*, a parasite naturally found in the lizard *Sceloporus ferrariperezi*, appears to lose its gametocytes completely when transferred to another lizard *Crotophytus collaris*. However, when the parasite is transferred to still another species, *Sceloporus olivaceus*, it regains its gametocytes. In this case, *Crotophytus collaris* as an environment suppresses temporarily the production of the sexual forms, but apparently, if Gambrell's thesis is right, does not affect the inheritance factors of the undifferentiated organisms in such a way as to prevent later gametocyte production in a more favorable host.

The change in strain 3H1-4 was apparently a true mutation in that it was an alteration of a more permanent nature. As W. H. Taliaferro and Huff (1940) point out, it is impossible to determine at the present time the genetic significance of these results, because of the inadequacies of our present methods

of study. If the concept advanced by Jollos (1921) is valid (that environmentally induced cytoplasmic changes can be transmitted over many asexual generations, although lost gradually in the absence of the causative stimulus, or suddenly as a result of endomixis or conjugation), then the changes produced in strain 3H1-3 would probably belong in this category. Sonneborn and Lynch (1934) and De Garis (1935), working with various types of sexual crosses in *Paramecium*, reported that cytoplasmic inheritance can continue under a different nuclear structure from the one originally determining the constitution of the cytoplasm. However, under the newly constituted nucleus, the character of the cytoplasm gradually changed. This fact indicates, as Jennings (1941) points out in his review, that there is at least some genetic basis for Jollos' hypothesis. Others, as is also pointed out by Jennings and by W. H. Taliaferro and Huff (1940), do not regard Jollos' concept as sound, but think that the reversion of the organisms to their original state is caused by the selective action of the original environment in favoring the reverse mutation. The temporary change in strain 3H1-3 lasted much longer than the types of "cytoplasmic lag" noted by the above workers on the free-living protozoa. The internal reorganizations in nuclear rearrangement that might exist in *Plasmodium* during the process of schizogony are unknown. For this reason it is impossible to state with surety whether the alteration of strain 3H1-3 was phenotypic or genotypic. The altered characteristic of strain 3D1 and 3H1-4 seems different in kind from those induced in strain 3H1-3. Both strains 3D1 and 3H1-4 have shown no tendency to revert to type, although they have been kept in an environment which caused strain 3H1-3 to revert.

It seems probable that in *P. cathe-merium* the hereditary factor which governs the ability to produce gametocytes is closely associated with the factor controlling the sharp synchronism. It is also probable that these factors are most subject to change when the organisms are exposed to severe environmental conditions. At least they are the only ones that have been affected up to the present time.

SUMMARY

1. The thermal death point of *Plasmodium cathe-merium* was determined to be 50 C for 8 minutes. A few parasites survived after exposure to this temperature for 7 minutes, but evinced a prolonged incubation period when inoculated into animals.

2. Three strains of *P. cathe-merium* (3H1-3, 3H1-4, 3H1-5) were isolated from strain 3H1-2 by means of exposure of the parasite to a temperature of 50 C,

3. At the time of the periodicity study, the parasites of strain 3H1-3 exhibited a marked disturbance of synchronism in the production of both asexual and sexual parasites. Seven months later this strain had returned to normal.

4. The parasites of strain 3H1-4 had almost lost their power of gametocyte production. There was a concomitant loss of periodicity in asexual reproduction. In the subsequent studies, this strain did not produce gametocytes and was incapable of infecting mosquitoes. Its asynchronism of the asexual reproduction remained unchanged.

5. The parasites of strain 3H1-5 demonstrated no change from the parent strain in the characteristics investigated.

6. Two effects of the crisis on the periodicity of the infection were: a. delay in segmentation, and b. a loss of synchronism during the crisis. These

effects, not necessarily concomitant, might occur separately or simultaneously. Similar effects were noted just before the death of the host. When the synchronism of the asexual cycle was disturbed by a crisis, the synchronism of the gametocyte production was also disturbed.

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